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MODES OF INFECTION OF THE ASCIDIAN *MOLGULA MANHATTENSIS* BY ITS ENDOSYMBIONT *NEPHROMYCES* GIARD

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Abstract

The renal sac of the sea squirt *Molgula manhattensis* consistently harbors a collection of fungus-like cells, "*Nephromyces*". These cells are not *Molgula* cells, but an organism(s) foreign to the host. *Nephromyces* does not have an obligate intermediate host. *Nephromyces* is not transmitted with the gametes of *Molgula*, but can be transmitted to *Molgula* through the ambient water. *Nephromyces* is released into the water after death of its host, although not necessarily only at this time. *Molgula* acquires *Nephromyces* after the initiation of feeding, which follows settling and metamorphosis. *Nephromyces* remains infective for at least twenty-nine days after isolation from its host.

Introduction

In the renal sac of all adults of all molgulid tunicate species so far examined (Saffo, 1982), fungal-like cells, *Nephromyces* Giard (1888), are present in conspicuous numbers.

The ubiquity of *Nephromyces* cells in the renal sac of these molgulids suggests that the interaction between *Nephromyces* and molgulids may be a symbiotic association with important effects on the activities of the renal sac and on the general biology of molgulids. To consider physiological and ecological aspects of such questions, it would be useful to understand the developmental relations between *Nephromyces* and its hosts. Are *Nephromyces* cells and molgulid tunicates associated with each other throughout the life cycles of both host and endosymbiont? How is *Nephromyces* transferred from its ductless habitat, the renal sac, to a new host? How is this association maintained, generation after generation?

The universality of the association between *Nephromyces* and at least six molgulid species also raises the most important question: is this really a symbiotic association, or is "*Nephromyces*" merely a collection of molgulid cells?

In considering some of the developmental interactions between *Nephromyces* and the ascidian *Molgula manhattensis*, this paper presents critical evidence for the existence of *Nephromyces*, and describes some means by which *Nephromyces* cells from *M. manhattensis* are transferred from host to host.

Materials and Methods

We collected adult *Molgula manhattensis* from San Francisco Bay, California and Vineyard Sound, Massachusetts.

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Nephromyces-*free* Molgula (*experiments* 1–5)

In the laboratory, field-collected adults were rinsed of debris and ambient water, then induced to spawn into 0.22 μm-filtered seawater (Saffo, 1978). Embryos were maintained in 0.22 μm-filtered seawater through metamorphosis and initiation of feeding by the settled zooids (Fig. 1). California-raised *M. manhattensis* were grown beyond this stage of development in coarsely-filtered (filtrate with particle size ≤ 1 mm) seawater collected from Bodega Bay, California, where *Molgula* (and *Nephromyces*) are not present. Massachusetts-raised *M. manhattensis* were grown in 0.8 μm-filtered seawater. These laboratory-grown *M. manhattensis* were fed a variety of axenically cultured unicellular algae, including *Dunaliella, Platymonas, Isochrysis, Monochrysis, Stichococcus*, and *Nannochlorus* spp.

Inoculation experiments

For experiments 1–4, laboratory-raised, *Nephromyces*-free *M. manhattensis* zooids were inoculated with *Nephromyces* by introduction of renal sac contents (including renal sac fluid, and some concretions, as well as *Nephromyces* cells) freshly dissected from sexually mature, field-collected *M. manhattensis*, into the ambient water of the settled zooids. After varying periods of exposure to *Nephromyces* cells (1 hour to 9 days at 16–19°C), culture dishes on which zooids had settled were rinsed thoroughly with 0.22 μm-filtered seawater.

In experiment 3, other modes of inoculation were tested as well. Some laboratory-raised, *Nephromyces*-free zooids were incubated at 17°C with living, *Nephromyces*-infected *M. manhattensis* (7–9 days in a one gallon aquarium with one or two field-collected adults; 54 days in a five gallon aquarium with 20 laboratory-

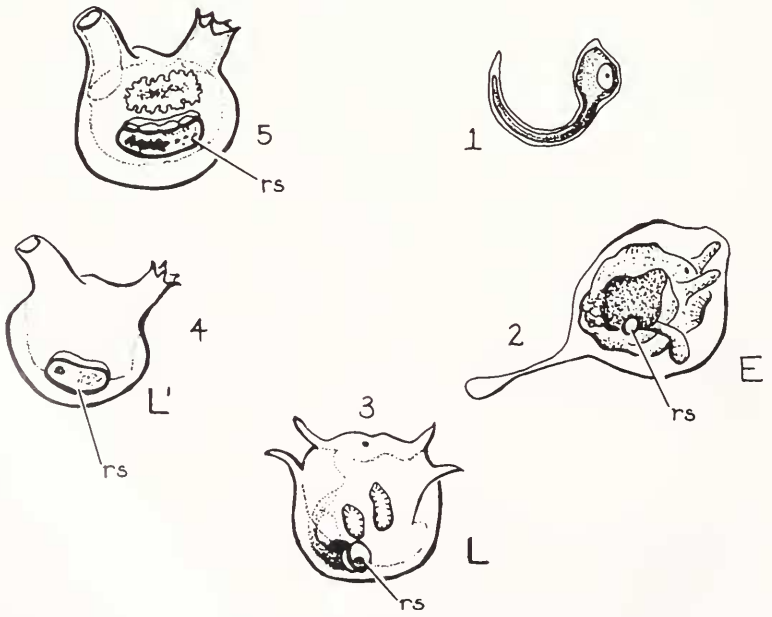

FIGURE 1. Inoculation of *Molgula* with *Nephromyces* before ("early": E) and after ("late": L, L') initiation of feeding. 1: tadpole larva; 2: settled, metamorphosing zooid (renal sac present, feeding organs not functional); 3: feeding zooid (5–7 days after fertilization); 4: zooid several months after fertilization; 5: sexually mature adult. *rs*, renal sac. Drawings not to scale.

raised zooids). Other *Nephromyces*-free zooids were settled onto slides, marked and introduced into San Francisco Bay in a crab trap one meter below the underside of a float in the Berkeley municipal marina. Large aggregations of (field-settled) *M. manhattensis* were present on this float. One group of such laboratory-settled zooids was suspended in the bay for five days in April 1978 (water temperature: 18°C). A second group was suspended in the bay for 13 days in July 1978 (water temperature: 20°C).

Each set of inoculation experiments included an uninoculated, control culture of laboratory-raised *M. manhattensis*.

Persistence of infectivity of Nephromyces *cells in seawater* (experiment 5)

Freshly isolated *Nephromyces* cells were centrifuged (20 min, 4°C, 900 *g*.) and, after decanting the renal sac fluid supernatant, resuspended in autoclaved, 0.22 μm-filtered seawater containing penicillin and streptomycin (0.25 g/l each). Such cell suspensions were added as inocula to cultures of *M. manhattensis* (feeding zooids) one, two, four and twenty-nine days after suspension in seawater, and rinsed from *M. manhattensis* cultures after eight hours' incubation at 19°C, or twenty-four hours' incubation at 16°C. A seven-day-old seawater suspension of such *Nephromyces* was filtered through a 5 μm Nuclepore filter, the filtrate then incubated with a *Nephromyces*-free *M. manhattensis* culture for 24 hours at 19°C.

After twelve or more days, *Nephromyces*-treated and control cultures of *M. manhattensis* were checked for the presence of *Nephromyces* in the renal sac lumen by examination of the transparent renal sac with phase contrast optics at 200–400×.

Results and Discussion

Experiment 1: Are *Nephromyces* cells passed on to the next generation of *Molgula manhattensis* with the gametes of the adult oviparous ascidian? Of the *M. manhattensis* raised from naturally-spawned eggs and sperm in *Nephromyces*-free seawater (ten separate experiments), none of the 266 zooids examined were infected with *Nephromyces*. Thus, *Nephromyces* is not transmitted with the sperm or eggs of *Molgula manhattensis*.

Field observations are consistent with these laboratory data. Although all adult *Molgula manhattensis* are infected with *Nephromyces* (Saffo, 1982), the renal sacs of immature *M. manhattensis* are not invariably infected (Table I). Further,

Table I

Percent Nephromyces-*infected individuals among field-settled* Molgula *zooids (San Francisco Bay)*

Collection date	Sexually mature?	Size (mm)	N	% Infected	Smallest infected individuals (mm)	Minimum size (mm) 100% infection
I. 5/15/78	no	≤2 (0.3–2.0)	10	0		
	no	2–6	22	23		
	no	6–10	9	44	3.5	≥13
	no (<13 mm); yes (≥13 mm)	10–14	5	80		
	yes	>14 (17–33)	11	100		
II. 6/19/78	no	≤2 (1.5–2.0)	4	25		
	no	2–8	4	100	1.5	≥2.5
	yes	>8 (20–30)	12	100		
III. 8/13/78	no	≤2 (0.8–2.0)	10	100	0.8	≥0.8
	no	>2 (4.0–10.5)	3	100		

the rate of infection of young *M. manhattensis* varies with season. Of 57 *M. manhattensis* (0.3–33.0 mm in diameter) collected from Berkeley marina (San Francisco Bay) early in the reproductive season (May 1978), only the renal sacs of sexually mature animals (diameter > 13 mm) were always infected with *Nephromyces*. No individual smaller than 3.5 mm possessed an infected renal sac. Of 20 *M. manhattensis* (1.5–30 mm in diameter) collected from the same location in June, 1978, all individuals larger than 2 mms were infected. In August, even the smallest zooids collected from San Francisco Bay (0.8 mm) were infected with *Nephromyces*. If *Nephromyces* were transmitted with the gametes of *M. manhattensis*, such seasonal differences in infection rate would not be expected.

Experiment 2: Does *Nephromyces* require passage through a second host before reinfection of *M. manhattensis*? As Tables II, III, and IV show, *Molgula* are infected with *Nephromyces* by addition of these cells to ambient seawater. Although *Nephromyces* may infect organisms other than molgulids, these experiments do indicate that *Nephromyces* does not absolutely require such an alternate host before successful reinfection of *M. manhattensis*.

Experiments 1 and 2: Are *Nephromyces* really *Molgula* cells, or, as their morphology suggests (Saffo, 1982), are they an organism(s) foreign to *Molgula*? If *Nephromyces* cells were merely tunicate cells, one would expect to always see *Nephromyces* cells in the renal sac, no matter how *M. manhattensis* were raised. Instead (experiment 1), *Nephromyces*-free *M. manhattensis* can be obtained routinely. Furthermore, the lumen of an uninfected renal sac is free not only of *Nephromyces* cells, but of *all* cells—making misinterpretation of such observations virtually impossible. This *Nephromyces*-free condition persists in all uninoculated

TABLE II

Modes of transmittance of Nephromyces *from host to uninfected zooids*

Mode inoculation	Control # (this table)	Time of inoculation (days after fertilization)	Period of inoculation (days)[c]	Size of inoculum	Time of examination (mos. after inoculation)	N examined	% Infection
1. — (Control)	—	—	—	—	2	32	0
2. — (Control)	—	—	—	—	4	20	0
3. — (Control)	—	—	—	—	4	21	0
4. — (Control)	—	—	—	—	6	9	0
5. — (Control)	—	—	—	—	9	7	0
6. — (Control)	—	—	—	—	1	26	0
7. Living infected adult	1	53	7	1 (25 mm) animal/1 liter	1	19	0
8. Living infected adult	2	2	9	2 (20 mm) animals/3 liters	3, 4	17	0
9. Field (S.F. Bay)	1	4/9/78; 4	5	—	2	26	0
10. Field (S.F. Bay)[b]	5	7/5/78; 0 (field-settled) + 257 (lab-settled)	13	—	1	23 (17 lab/6 field)	100
11. Infected Laboratory Zooids[b]	1	113	54	20 (2 mm) animals/21 liters	2	20	100
12. Renal Sac contents[a,b]	2	2	9	2 renal sac/1 liter	2, 4	23	100
13. Renal Sac contents[a,b]	3, 4	173	9	2 renal sac/1 liter	6	3	100
14. Renal Sac contents[a,b]	6	6	1	2 renal sac/1 liter	1	16	100

[a] See also tables 3 & 4 for shorter inoculation periods.

[b] Inoculum included dead animals (or portions of dissected animals).

[c] Temperature = 17–20°C in all cases.

TABLE III

Percent Nephromyces-*infected individuals among laboratory-raised* Molgula *infected "early" (before siphons open) and "late" (after siphons open) in metamorphosis (Figure 1)*

Trial #	Time of inoc. after fertilization (days)	Period of inoculation (hours)/ temperature	Time of examination after fertilization (days)	N Examined	% Infected
1	C:[a] —	—	24	25	0
	E:[a] 1.5	1/(19°C)	24	27	0
	L:[a] 4	1/(19°C)	24	25	100
2	C: —	—	23	25	0
	E: 2	1/(19°C)	24	28	3.6
	L: 4	1/(19°C)	22	25	100
3	C: —	—	26	25	0
	E: 2	1/(15°C)	31, 36, 94	28	0
	L: 7	1/(15°C)	26–31	27	63

[a] C = control; E = early; L = late.

M. manhattensis, no matter what the general condition of the animals themselves, or the contents (other than absence of *Nephromyces* cells) of ambient water. If *Nephromyces* cells were really tunicate cells that appeared only in certain environmental situations, one would expect to see some exceptions to these results. If, for instance, *Nephromyces* cells were *Molgula* cells which arose only in response to microbial infection, one would expect that the renal sac of *M. manhattensis* raised in coarsely-filtered (but *Nephromyces*-free) seawater, which can contain a variety of protists, fungi and small metazoans, might occasionally be infected with *Nephromyces* cells—but they are not. Likewise, one might also expect unhealthy (clogged intestine, feeble siphon response, infestations of ciliates or bacteria in the tunic), uninoculated *Molgula* to possess *Nephromyces* cells—but they do not. In short, *M. manhattensis* contain *Nephromyces* cells only if the *Molgula* cultures

TABLE IV

Infectivity of Nephromyces *after isolation from the renal sac*

Nephromyces inoculum	Days of axenic incubation of *Nephromyces* after isolation from renal sac	Period of inoculation (hrs.)	Date of inoculation (days after fertilization of M. manhattensis)	Date of examination (days after fertilization/ inoculation	N Examined	% Infection
1[a]—(Control)	—	—	—	24/—	25	0
2[a] +	1	8	32	57/25	25	100
3[a] +	2	8	33	58/24	25	100
4[a] +	4	8	35	58/23	25	100
5[a] +	7	24	38	58/20	25	100
6[b] +	29	24	6	18/12	32	100

[a] inoculation/incubation temperature: 19°C.
[b] Inoculation/incubation temperature: 16°C.

have been specifically inoculated with *Nephromyces* cells. Thus, *Nephromyces* cells are not *Molgula* cells; they are cells foreign to the tunicate.

Experiment 3: How are *Nephromyces* cells transmitted from the ductless renal sac to a new host? Experiment 1 indicates that *Nephromyces* is not released with the gametes of *M. manhattensis* at spawning. Experiment 2 indicates that *M. manhattensis* can be infected by *Nephromyces* released directly into seawater after dissection from the renal sac. In natural conditions, are *Nephromyces* cells released from *Molgula* only in a similar event (death of the host), or are they released from living animals as well? One month after 7–9 days' incubation of laboratory-raised, *Nephromyces*-free *M. manhattensis* zooids with living, field-collected, *Nephromyces*-infected *M. manhattensis*, none of the lab-raised *M. manhattensis* had become infected (Table II). After 54 days' incubation of lab-raised *Nephromyces*-free zooids with lab-raised, *Nephromyces*-infected zooids, all of the formerly *Nephromyces*-free zooids had become infected. *Nephromyces* may be released infrequently, or in small numbers from living *Molgula*, such that only lengthy exposure of the inoculum results in 100% infection of young zooids. Or, since several *Nephromyces*-infected zooids died in the aquarium during the course of the long-term incubation, *Nephromyces* may be released only from dead *Molgula*. Our laboratory experiments do not distinguish between these two alternatives, but the following observations favor the latter possibility.

In early April, a group of lab-raised *Nephromyces*-free zooids was suspended in San Francisco Bay for five days. Extensive aggregations of "wild" *M. manhattensis* (only a few of them sexually mature adults) were located one meter away on adjacent floats. Eight weeks later, none of the lab-raised zooids were infected with *Nephromyces*. In early July, a second group of such lab-raised zooids was suspended in the bay for 13 days, again with extensive aggregations of *M. manhattensis* (this time virtually all of them adults) on neighboring floats, and with several dead *M. manhattensis* floating in the water within a meter of the lab-raised zooids. Three weeks after removal from the bay, all lab-settled *M. manhattensis* (6–10 mm diameter) and young field-settled *M. manhattensis* (1–2 mm diameter) were infected with *Nephromyces*. Although the difference in incubation time in the two field trials may be partly responsible for the differences in infection rates, both the striking difference of rate (0% *vs.* 100%) and the short time required for infection in the laboratory (40–60 minutes, experiment 4) suggest that other factors—especially seasonal differences in numbers and death rate of nearby adult *M. manhattensis*—might be chiefly responsible for the differences in infection rate.

None of these experiments eliminate the possibility that *Nephromyces* cells are released from living *M. manhattensis*. However, in all the successful inoculation trials–and only in these trials—dead infected *M. manhattensis* were clearly part of the potential source of infection. This is consistent (as is our standard laboratory infection procedure) with the possibility that, in nature, *Nephromyces* cells are released into the ambient water only upon death of their tunicate host.

Experiment 4: How does *Nephromyces* enter a new host? *M. manhattensis* exposed to *Nephromyces* at different stages of development—after settling and formation of the renal sac but before the siphons open (Fig. 1, "early inoculation," Saffo, 1978), and after the siphons open (the beginning of feeding, "late inoculation")—show striking differences in infection rate (Table III). In two of the three trials (Table III), none of the *M. manhattensis* inoculated "early" in metamorphosis were infected with *Nephromyces* 3–13 weeks after inoculation. In a third trial, only a single *M. manhattensis* (out of 28 examined) was infected. In contrast, 63–100% of the *M. manhattensis* inoculated after completion of metamorphosis were infected

with *Nephromyces* a month after inoculation. This indicates that *Nephromyces* enters *Molgula* most readily through the siphons of the host, presumably most easily through the incurrent siphon, in feeding. While the single infected "early-inoculated" *Molgula* may have become infected by *Nephromyces* in some other manner (*e.g.*, penetration of the tunic), results of experiment 5 (below) are consistent with the hypothesis that *Nephromyces* might occasionally survive post-inoculation rinsing, persist on the surface of *Molgula* or the culture dish, and later be ingested by *Molgula* zooids after they start feeding.

This experiment establishes the developmental stage at which *Molgula* is ingested, and the general way in which *Nephromyces* enters its host. It does not establish, however, how *Nephromyces* enters the ductless renal sac; the latter process will be described elsewhere (Saffo, unpublished).

Experiment 5: *Nephromyces* cells are not transmitted from *Molgula* with gametes of the host. Rather, they are transmitted through the ambient water—perhaps only from dead, ingested *Molgula* and probably to new *Molgula* only via feeding currents. How can such an apparently haphazard mode of transmission lead to 100% infection of adult *M. manhattensis* by *Nephromyces*—especially early in the reproductive season, when the numbers of adult *Molgula* (the ultimate source of *Nephromyces*) are low?

M. manhattensis can be readily infected with *Nephromyces* immediately after initiation of feeding, and also long after completion of metamorphosis (Table II). Laboratory-raised animals were successfully inoculated as long as 9 months after fertilization; the largest such animal (L′, Fig. 1) was 77 mm in diameter, and showed initial traces of gonad formation (gonoduct, immature oocytes, *etc.*). Patterns of infection of *M. manhattensis* in the field (Table I) are consistent with these experimental data. The susceptibility of *Molgula* to infection by *Nephromyces* for a long developmental period after the initiation of feeding must extend the chances for infection.

These chances are also favored because at least some *Nephromyces* cells can retain their infectivity long after isolation from their host. Exposure of *M. manhattensis* zooids to *Nephromyces* incubated one, two, four, seven or twenty-nine days in seawater (Table IV) invariably results in 100% infection of *Molgula.*

This experiment, as well as experiments 1 and 4, indicate that *Molgula* and *Nephromyces* can survive independently for extended periods. Certainly, neither organism is associated with the other throughout its entire life cycle. Are *Molgula* and *Nephromyces* nevertheless dependent on each other for completion of their life cycles? Since the *Nephromyces* cell which survives longest in seawater may be a resistant spore (Saffo, unpublished), the survivability of *Nephromyces* outside *Molgula* is still compatible with the notion that infection of *Molgula* is an obligate portion of *Nephromyces*' life cycle.

The dependence of *Molgula* on *Nephromyces*, however, is more problematic. Since *Molgula* does not acquire *Nephromyces* until after metamorphosis, *Molgula's* dependence (if any) on *Nephromyces* must be in some aspect of post-metamorphic metabolism, growth or development.

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